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1. Your reference JB/HD/01

2. Patent application number *(The Patent Office will fill in this part)* 9923381.9

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Jan Joris Brosens
29 Earthew Road, London W6 0DU
UK

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

THE USES OF CELL-SPECIFIC SPLICING IN GENE THERAPY

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

| Country | Priority application number *(if you know it)* | Date of filing *(day / month / year)* |
|---|---|---|

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

| Number of earlier application | Date of filing *(day / month / year)* |
|---|---|

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

*a) any applicant named in part 3 is not an inventor, or*
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| Continuation sheets of this form | |
| Description | 6 |
| Claim(s) | 2 |
| Abstract | 1 |
| Drawing(s) | 3 |

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|---|---|
| Priority documents | |
| Translations of priority documents | |
| Statement of inventorship and right to grant of a patent *(Patents Form 7/77)* | |
| Request for preliminary examination and search *(Patents Form 9/77)* | 1 |
| Request for substantive examination *(Patents Form 10/77)* | 1 |
| Any other documents *(please specify)* | |

11. I/We request the grant of a patent on the basis of this application.

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12. Name and daytime telephone number of person to contact in the United Kingdom

0131-741 9706
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Background

With the advent of molecular biology has come the ability to express nucleotide sequences of interest (NOI) within desirable cell types. For gene therapy protocols often such expression requires targeting to ensure that the desired NOI is expressed only within the appropriate cells. To date such targeting is achieved by either 'physical' means or use of transcriptional control or both.

Physical targeting can involve purification steps such that the resulting NOIs are delivered, post-purification, to the an enriched cell fraction such as CD34+ cells of blood. Other physical means by which targeting is achieved can be by way of using targeted NOI delivery vehicles (e.g. viral vectors or synthetic complexes) designed such to only deliver NOIs to the appropriate cell-type.

Transcriptional targeting often involves either synthetic or natural promoter elements designed to limit NOI expression to the appropriate cell types/ and or appropriate environment.

Although such cell/environment-specific targeting has great potential for treatment of human diseases and disorders there are still difficulties. For physical targeting, the desired cell-types can not always be enriched prior to NOI delivery. Furthermore targeted delivery vehicles are often inefficient in delivering NOI only to the desired cell type. For transcriptional targeting a major problem is that the synthetic or natural promoter elements, designed to confer cell/environment-specific NOI expression, are often 'leaky' resulting in expression of the NOI in cells other than the desired environment/cell type.

Presented here is a novel way by which targeting might be achieved in a cell/environment-specific manner. This invention involves the use of cell-type specific splicing events to limit NOI translation to desired cell types. This targeting strategy can therefore be considered both post-NOI delivery and post-NOI transcription in gene therapy protocols. It can, however, also be used in conjunction with other targeting strategies if so desired.

Detailed outline of inv[redacted]n.

Although higher eukaryotic cells utilise promoter elements as the main method by which gene expression is restricted to desired cell types/environments, there are however other methods by such expression can be regulated. One such method is at the level of post-transcription, and involves alternative splicing between cell types such that cell-type specific spliced transcripts are made, producing cell-type specific translated gene products. A well characterised example is the regulation of rat B-tropomysin expression in muscle and non-muscle cells (see Balvay *et al* 1992 NAR 20: 3987-3992; Guo and Helfman 1993 NAR 21:4762-4768). In this case, exons 1-5, 8 and 9 are common to all mRNAs expressed from this gene. However in fibroblasts and smooth muscle cells exons 6 and 11 are used, whilst in skeletal muscle cells exons 7 and 10 are exclusively used. Studies of these splicing events have localised the critical elements for alternative splicing to sequences within exon 7 and the adjacent upstream intron. Moreover these sequences have been shown to confer cell type specific splicing when flanked with heterologous exons. For example Gou and Helfman (*i.b.i.d)* have shown that the rat B-tropomycin exon 7 and its flanking intronic sequences are sufficient to regulate the suppression of exon 7 in non-muscle cells even when flanked by heterologous exons derived from adenovirus.

In part this invention relates to the use of these exon7/upstream intron regulatory sequences to restrict of gene expression to skeletal muscle cells. Outlined in **Figure 1A** is a representative diagram of a vector, in this instance pCI (Promega®) based, in which the pCI intron related sequences are replaced with the splice donor/flanking sequence (50 base pairs either side of the splice donor) from exon 5 of the rat B-tropomysin gene and the splice acceptor/flanking sequence (from upstream of the branchpoint through 25 nucleotides downstream of exon 7) of the rat B-tropomycin gene. Between these two sequences is inserted a luciferase gene taken from pGL3 (Promega®) with its open reading frame (ORF) orientated as shown. Downstream of all these sequences is located an enhanced green fluorescence protein (EGFP) ORF (Clontech®) as shown in **Figure 1A**. The poly A signal is as found downstream of the multiple cloning site of pCI. To ensure translation begins from the desired ATG (of either luciferase or

EGFP), upstream ATG sequences present in the primary transcript are mutated (in this instance by ATG to ACG mutagenesis). Such a vector can be made by established methods by those skilled in the art.

Because of the blockage in splicing conferred by exon 7 sequence in non-skeletal muscle cells, the resulting vector will produce unspliced transcripts in non-skeletal muscle cells (**Figure 1B**) and spliced transcripts in skeletal muscle cells (**Figure 1C**). As a consequence of this design, in skeletal muscle cells there is observed an increase in EGFP expression and a decrease in luciferase expression. In non-skeletal muscle there is instead an observed increase in luciferase expression and a decrease in EGFP expression. Consequently, in this example EGFP has skeletal muscle-specific high expression and luciferase has non-skeletal muscle-specific high expression. Of note is that although when unspliced, EGFP is still present within the RNA transcript, without the use of internal ribosome entry site sequences, ribosome translation will only lead to luciferase translation in a 5'-cap-dependent manner.

This example therefore demonstrates the potential for the use of *cis*-elements to limit expression of exogenous genes to specific cell-types. Although in this instance marker/reporter genes are used, these examples are not limiting. Other NOI of interest or of therapeutic value could be used instead.

This invention is however not limited to the use of intronic elements of the rat B-tropomysin gene but this was shown by way of example of how exactly such an invention works. Aberrant or alternative splicing of specific mRNAs is a common occurrence in human malignancies and the products of such aberrant splicing events have been implicated in the oncogenesis and progression of human tumours. For instance malignancy associated aberrant/alternative splicing has been observed for the RNA message for TSG101 (Lin *et al* 1998 Br J. Haematol. 1998 102: 753-8: Lee and Feinberg 1997 Cancer Res. 57; 3131-4 ), cathepsin B (Mehtani *et al* 1998 J Biol Chem. 273 13236-44), epidermal growth factor receptor (eg. see Yamazaki *et al* J. Natl. Cancer Inst. 1998 90: 581-7; Sugawa *et al* 1990 PNAS: 87:8602-6), CD44 (see Kittl *et al* 1997 Exp. Clin. Immunogenet 14; 264-72; Sherman *et al* 1997 Cancer Res 57; 4889-97), NER (Saito *et al*

Oncogene 1997 14: [redacted]21), platelet-derived growth factor [redacted]eptor (Mosselman *et al* 1996 PNAS 93; 2884-8), stem-cell factor receptor (Takaoka *et al* 1997 Cancer Lett. 115; 257 glucocorticoid receptors (Moalli and Rosen 1994 Leuk Lymphoma 15: 363-74), and p53 (Nakai *et al* 1994 Br. J. Haematol.). Often aberrant/alternative splicing of certain transcripts in human diseases is highly specific and the presence of these aberrant/alternative spliced transcripts can be used as molecular markers for the diagnosis of emerging or residual disease (see Taniguchi *et al* US patent 5643729 (assignees Boehringer Ingleheim))

An example of how such aberrant/alternative splicing events in human diseases can be exploited for transfer of NOIs in a cell-specific manner will now be outlined.

Progression of astrocytes from benign to a malignant phenotype (glioblastoma) is accompanied by a change in the RNA processing of fibroblast growth factor receptor-1 (FGFr) gene. The level of the high affinity form of the FGFr is dramatically increased due to α-exon skipping during RNA splicing. The *cis*-regulatory sequence that confer this exon skipping have been elucidated (Cote *et al* 1997 J Biol. Chem. 272:1054-1060) by use of a vector construct termed pFGR17 (Cote *et al* 1997 *i.b.i.d.)*. This construct was originally made by cloning the α-exon, γ-exon and flanking sequence into intron 1 of the human metallothionein (hMT) gene. Consequently, transcripts produced from this vector, when spliced, have hMT exon-1, α-exon, and hMT exon-2 of the mRNA joined. However if the inserted FGFr sequence induces exon skipping then α-exon sequence would be exluded in the final mRNA message. Subsequent stepwise deletion of the inserted sequence demonstrated that a 375 nucleotide sequence inclusive and flanking that of the α-exon was able to confer the exon skipping phenotype, provided that the overall insert size was maintained. These sequences had splice-functional *cis*-elements involved someway in altering splicing in glioblastoma cells.

The pFGR-17 vector used to map the *cis* elements involved in FGFr exon skipping is the starting construct of this aspect of the invention- namely to generate glioblastoma-specific NOI translation/expression by conditional splicing (**Figure 2A**). Into the vector is first cloned a NOI, in this case the EGFP marker. This NOI is cloned downstream of the splice acceptor related

sequences of hMT-2 exo[redacted] pFGR-17. Next, to ensure that u[redacted] splicing, translation starts from EGFP ATG and not upstream ATGs found either within hMT-1 and hMT-2 sequence, such upstream ATGs are disabled by site-directed mutagenesis of ATG to ATTG. This vector can be made by established methods by those skilled in the art.

In this instance the presence of ORFs within the α-exon / flanking regions ensures that unless exon skipping occurs, EGFP is never translated. However in glioblastomas, α-exon induced skipping leads to upstream ORF removal and subsequently EGFP is then the first ORF of the transcript and translated as such. The process of exon skipping is restricted to malignant cells and hence EGFP expression is confined to glioblastoma cells (**Figure 2C**) and fails to occur in healthy astrocytes (**Figure 2B**). Although in this instance a marker gene (EGFP) is used, this examples is not limiting. Other NOI of interest or of therapeutic value could replace EGFP, using established methods by those skilled in the art.

These two examples demonstrate how the use of aberrant or alternative splicing can be exploited to restrict gene expression to certain environments and cell types. They aid in understanding the current invention but the invention is not limited to the use of these two preferred embodiments. The use of this invention include, but are not limited to, *in-vivo* and *ex-vivo* therapeutic regimes as currently undertaken in gene therapy and gene delivery protocols.

For the development of other cell-type specific splicing vectors in manners similar to that described above, the *cis*-elements in the transcript that confer cell-specific splicing may require characterisation. This can be done prior to construction of the cell-type splicing specific NOI-delivery vehicle by methodology known in the art; by way of example methodology see Cote *et al* (1997 J Biol. Chem. 272:1054-1060), Balvay *et al* (1992 NAR 20: 3987-3992) and Guo and Helfman 1993 (NAR 21:4762-4768).

Alternatively, cell-type specific splicing elements can be identified by a novel approach using original genomic DNA libraries. By way of example (**Figure 3**), a gene expression screen is developed whereby a selectable marker is placed within an intron and, hence, will not expressed

unless splicing is inh[redacted]d. Downstream of the selectable [redacted]ker is then cloned a sheared genomic library. The library is then introduced into a desirable cell population, desirable stimulus performed and subsequent marker expressing cells identified. Desirable stimuli might include mitogen/oncogene stimulation of the cell population prior to selection such that sequences are identified within positive cells that confer mitogen-specific splicing inhibition.

Claims

1 The use of rat B-tropomycin exon 5 splice donor (and surrounding functional sequence) and/or the use of rat B-tropomycin exon 7 splice acceptor (and surrounding functional sequence) to confer cell specific splicing events that limit optimal NOI translation/expression to either skeletal muscle or non-muscle cells. The NOI delivery vector maybe of any type: Plasmid-based vectors, retroviral based vectors, lentiviral-based vectors, adenoviral based vectors and combinations of such are example such NOI delivery vectors. The promoter and polyadenylation signals used to mediate the transcription of the RNA transcripts as described can be of any origin.

2 The use of B-tropomycin exon 5 splice donor (and surrounding splice-functional sequence) from any species and/or the use of B-tropomycin exon 7 splice acceptor (and surrounding splice-functional sequence) from any species to confer cell specific splicing events that limit optimal NOI translation/expression to either muscle or non-muscle cells. The NOI delivery vector maybe of any type: Plasmid-based vectors, retroviral based vectors, lentiviral-based vectors, adenoviral based vectors and combinations of such are examples. The promoter and polyadenylation signals used to mediate the transcription of the RNA transcripts as described can be of any origin.

3 The use of human FGFr α-exon (and surrounding splice-functional sequence) to confer cell specific splicing events that limit optimal NOI translation/expression to glioblastoma related/derived cells. The NOI delivery vector maybe of any type: Plasmid-based vectors, retroviral based vectors, lentiviral-based vectors, adenoviral based vectors and combinations of such are example. The promoter and polyadenylation signals used to mediate the transcription of the RNA transcripts as described can be of any origin.

3 The use FGFr α-exon (and surrounding splice-functional sequence) from any species to confer cell specific splicing events that limit optimal NOI translation/expression to glioblastoma related/derived cells. The NOI delivery vector maybe of any type: Plasmid-based vectors,

retroviral based vecto[redacted]ntiviral-based vectors, adenoviral b[redacted] vectors and combinations of such are examples. The promoter and polyadenylation signals used to mediate the transcrip of the RNA transcripts as described can be of any origin.

5 The use of any invention according to claims 1-4 whereby removal/mutation of any unrequired upstream ATG translation initiation sites is used to ensure translation initiation at the desired ATG such that of the NOI translation/expression is optimal.

6 The use of other splice sites and surrounding splice-functional elements from any origin (either natural or synthetic) in a manner and configuration similar to that described for examples 1 and 2 of the text to confer cell type specific splicing such that NOI optimal translational/expression is restricted to specific cell types and/or environment.

7 The use of libraries as outlined in the text to identify sequence elements capable of conferring cell specific splicing of desirable nature.

8 The use of the invention according to claims 1-7 when used to in conjunction with other methodologies, protocols and regimes for the treatment of diseases and disorders.

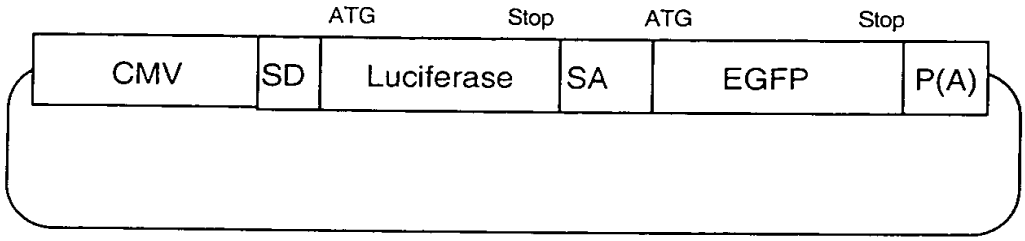
# The uses of cell-specific splicing in gene therapy

## Abstract

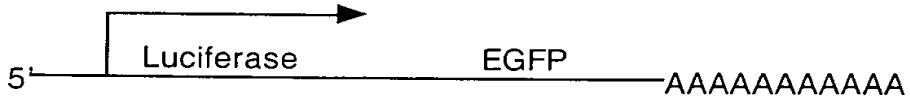
This invention relates to the construction of eukaryotic expression vectors. In particular this invention relates to the use of a novel methodology to regulate cell/environment-specific expression of such vectors. More particularly, this invention relates to the use of splicing elements within such vectors to restrict desired gene translation/expression to desired environments/cell types in the treatment of human diseases and disorders.

Figure 1

(A)


ATG
Stop
ATG
Stop
CMV
SD
Luciferase
SA
EGFP
P(A)

(B)


5'
Luciferase
EGFP
AAAAAAAAAAA

(C)

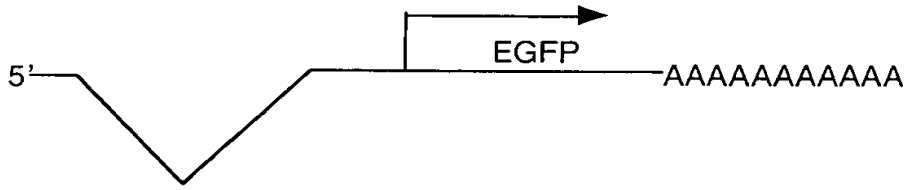
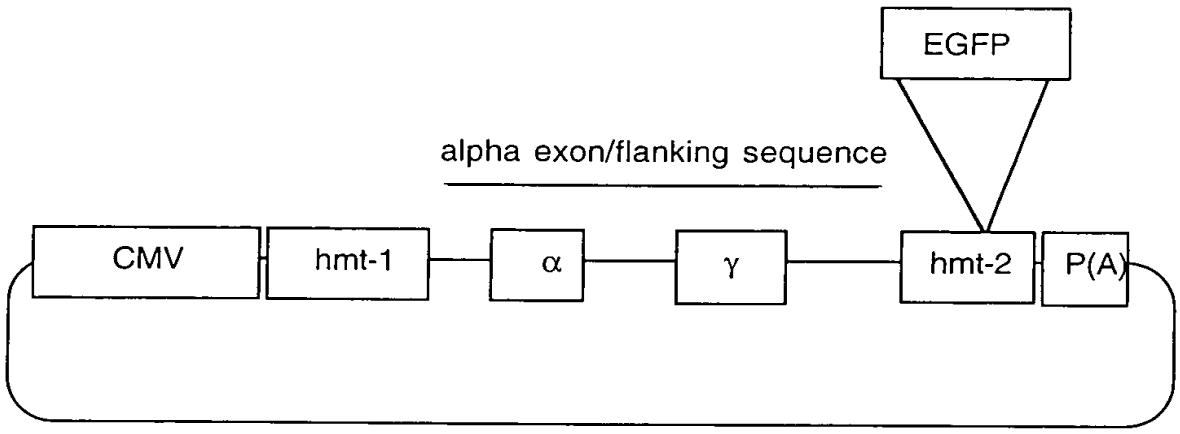
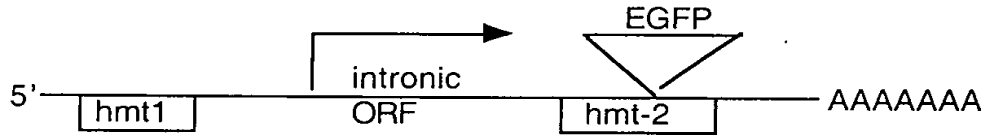

5'
EGFP
AAAAAAAAAAA

Figure 2

(A)


EGFP
alpha exon/flanking sequence
CMV
hmt-1
α
γ
hmt-2
P(A)

(B)


EGFP
intronic
ORF
5'
hmt1
hmt-2
AAAAAAA

(C)

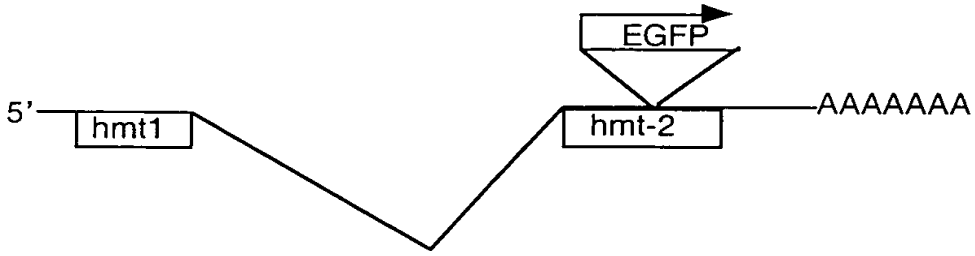
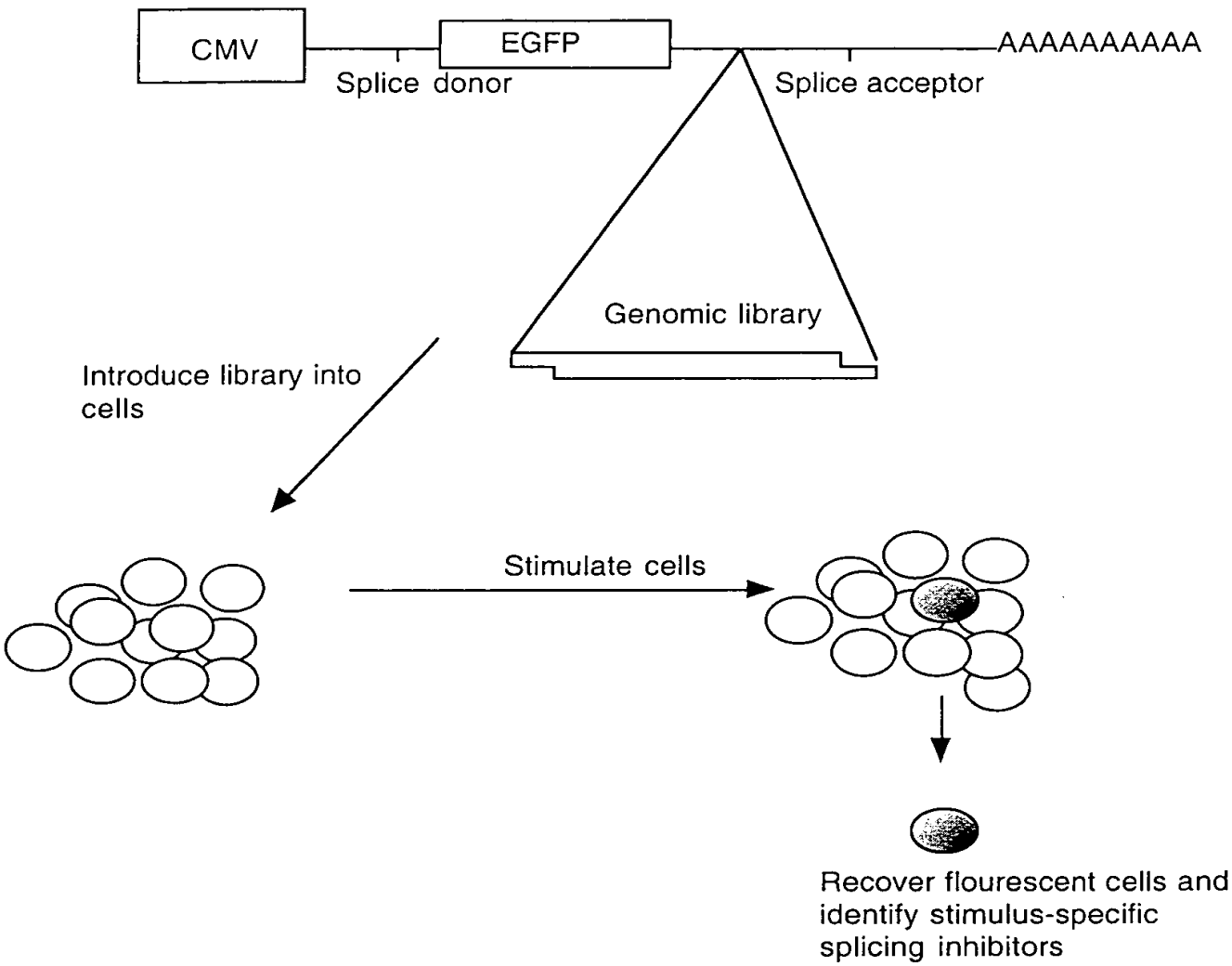

EGFP
5'
hmt1
hmt-2
AAAAAAA

Figure 3


CMV
EGFP
AAAAAAAAAA
Splice donor
Splice acceptor
Genomic library
Introduce library into cells
Stimulate cells
Recover flourescent cells and identify stimulus-specific splicing inhibitors

PCT / GB00 / 03806

Frank B Dehn & Co.

5/10/00

(Tracey Carter)